

## PATENT COOPERATION TREATY

PCT

## NOTIFICATION OF ELECTION

(PCT Rule 61.2)

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|   |   |
|---|---|
| Date of mailing (day/month/year)<br>04 August 1997 (04.08.97)             |   |
| International application No.<br>PCT/NO96/00266                           | Applicant's or agent's file reference<br>Eij 1 HV             |
| International filing date (day/month/year)<br>13 November 1996 (13.11.96) | Priority date (day/month/year)<br>13 November 1995 (13.11.95) |
| Applicant<br>EIJSink, Vincent, G., H. et al                               |   |

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International Preliminary Examining Authority on:

03 June 1997 (03.06.97)

☐ in a notice effecting later election filed with the International Bureau on:2. The election ☒ was☐ was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

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# INTERNATIONAL SEARCH REPORT

International application No.

PCT/NO 96/00266

## A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 15/74, C07K 14/335

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N, C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, FULLTEXT, MEDLINE, BIOSIS, DERWENT BIOTECH ABS, EMBL/GENBANK/  
SWISSPROT/DDBJ

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No. |
|-----------|--|-----------------------|
| P,X       | Molecular Microbiology, Volume 18, No 4, 1995,<br>Dzung Bao Diep et al, "A bacteriocin-like peptide<br>induces bacteriocin synthesis in Lactobacillus<br>plantarum C11" page 631 - page 639<br><br>--                                  | 1-15,17-21            |
| X         | Microbiology, Volume 140, 1994, Petra S. Tichaczek<br>et al, "Cloning and sequencing of sakP encoding<br>sakacin P, the bacteriocin produced by<br>Lactobacillus sake LTH 673", page 361 - page 367,<br>see especially fig 2<br><br>-- | 1-15,17-21            |

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

\* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search

13 February 1997

Date of mailing of the international search report

28 -02- 1997

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# INTERNATIONAL SEARCH REPORT

International application No.

PCT/NO 96/00266

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No. |
|-----------|--|-----------------------|
| X         | Molecular Microbiology, Volume 17, No 3, 1995,<br>Koen Venema et al, "Functional analysis of the<br>pediocin operon of <i>Pediococcus acidilactici</i><br>PAC1.0: PedB is the immunity protein and PedD is<br>the precursor processing enzyme",<br>page 515 - page 522, fig 1 and summary<br><br>--            | 1-15,17-21            |
| X         | Applied and Environmental Microbiology, Volume 60,<br>No 1, January 1994, Dzung Bao Diep et al, "The<br>Gene Encoding Plantaricin A, a Bacteriocin from<br><i>Lactobacillus plantarum</i> C11, Is Located on the<br>Same Transcription Unit as an agr-Like Regulatory<br>System" page 160 - page 166<br><br>-- | 1-15,17-21            |
| X         | Journal of Bacteriology, Volume 177, No 8, April<br>1995, Lars Axelsson et al, "The Genes Involved in<br>Production of and Immunity to Sakacin A, a<br>Bacteriocin from <i>Lactobacillus sake</i> Lb706"<br>page 2125 - page 2137<br><br>--  | 1-2,6-15,<br>17-21    |
| X         | Applied and Environmental Microbiology, Volume 57,<br>No 2, February 1991, Marco J. van Belkum et al,<br>"Organization and Nucleotide Sequences of Two<br><i>Lactococcal</i> Bacteriocin Operons",<br>page 492 - page 498, figure 1<br><br>--  | 1-2,6-15,<br>17-21    |
| X         | EP 0493779 A1 (QUEST INTERNATIONAL B.V.),<br>8 July 1992 (08.07.92), page 3, line 24 - line 31<br><br>--   | 1-2,6-15,<br>17-21    |
| X         | WO 9404682 A1 (DZIEGLEWSKA, HANNA, EVA),<br>3 March 1994 (03.03.94), page 11,<br>line 12 - line 37<br><br>--   | 1-2,6-15,<br>17-21    |
| X         | WO 119802 A1 (HOLMES, MICHAEL, J.),<br>25 December 1991 (25.12.91), page 6,<br>line 9 - line 36<br><br>--<br>-----   | 1-2,6-15,<br>17-21    |

**INTERNATIONAL SEARCH REPORT**  
Information on patent family members

International application No.  
PCT/NO 96/00266

| Patent document<br>cited in search report | Publication<br>date | Patent family<br>member(s) | Publication<br>date |
|---|---------------------|----------------------------|---------------------|
| EP-A1- 0493779                            | 08/07/92            | AU-B- 638616               | 01/07/93            |
|   |                     | AU-A- 8820091              | 09/07/92            |
|   |                     | CA-A- 2056086              | 01/07/92            |
|   |                     | JP-A- 7067652              | 14/03/95            |
|   |                     | US-A- 5175252              | 29/12/92            |
|   |                     | US-A- 5260212              | 09/11/93            |
| WO-A1- 9404682                            | 03/03/94            | AU-A- 4968893              | 15/03/94            |
| WO-A1- 119802                             | 25/12/91            | NONE                       |                     |

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

|   |  |  |  |
|---|--|--|--|
| Applicant's or agent's file reference<br>Eij 1 im   | <b>FOR FURTHER ACTION</b>                                |  | See Notification of Transmittal of International Preliminary Examination Report (PCT/IPEA/416) |
| International application No.<br>PCT/NO96/00266   | International filing date (day/month/year)<br>13/11/1996 | Priority date (day/month/year)<br>13/11/1996 |  |
| International Patent Classification (IPC) or national classification and IPC<br>C12N15/74 |  |  |  |
| Applicant<br>Eijsink, Vincent G.H. et Al.   |  |  |  |

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

☒ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 3 sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

|   |  |
|---|--|
| Date of submission of the demand<br>03/06/1997  | Date of completion of this report<br>24.02.98                              |
| Name and mailing address of the IPEA/<br><br>European Patent Office<br>D-80298 Munich<br>Tel. (+49-89) 2399-0. Tx: 523656 epmu d<br>Fax: (+49-89) 2399-4465 | Authorized officer<br><br>Huber, A<br><br>Telephone No. (+49-89) 2399-8173 |



**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/NO96/00266

**I. Basis of the report**

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

**Description, pages:**

1-27 as originally filed

**Claims, No.:**

1-15 with telefax of 16/02/1998

**Drawings, sheets:**

1/3-3/3 as originally filed

2. The amendments have resulted in the cancellation of:

- ☐ the description, pages:  
☐ the claims, Nos.:  
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/NO96/00266

**V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

**1. Statement**

|                               |      |        |      |
|-------------------------------|------|--------|------|
| Novelty (N)                   | Yes: | Claims | 1-15 |
|                               | No:  | Claims |      |
| Inventive step (IS)           | Yes: | Claims | 1-15 |
|                               | No:  | Claims |      |
| Industrial applicability (IA) | Yes: | Claims | 1-15 |
|                               | No:  | Claims |      |

**2. Citations and explanations**

**see separate sheet**

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

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International application No. PCT/NO96/00266

SECTION V -----

1. The present application relates to an inducible gene expression system in lactic acid bacteria. It has been discovered that expression of the IF-K-R gene cluster is autoinduced by the gene product of the IF gene.

2. The quoted documents are:

D1: P. Tichaczek et al., Microbiology, Vol. 140, pp. 361-367, 1994

D2: Axelsson et al., Journal of Bacteriology, Vol. 177, No. 8, pp. 2125-2137, 1995

3. D1 discloses the sequence of bacteriocin SakP, its promoter and two further open reading frames (orfX and orfY), whose function is unknown. It is speculated that these ORFs could encode immunity proteins.

D2 is concerned with an operon containing the genes necessary for production and immunity of the bacteriocin sakacin A (SapA) from *Lactobacillus sake* Lb706. It is assumed that proteins SapK and SapR constitute a two-component signal-transduction system, mediating a response to an environmental signal, while SapT and SapE seem to be involved in secretion of SapA. SaiA encodes the immunity peptide. Two further open reading frames were identified (Orf4 and Orf1) but no function has been accorded to them. Both are similar to the bacteriocin leader peptides and are speculated to be structural genes for bacteriocins.

4. Claim 1 relates to a gene expression system in lactic acid bacteria which is characterised in that it comprises a gene/genes of interest which have been operably linked by genetic engineering to a strongly regulated promoter whose activity can be induced by an unmodified peptide. The promoter elements and the

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

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International application No. PCT/NO96/00266

peptide are defined in the claim by all essential elements thereof. It is also specified in the claim that the genes of interest are **not identical** to the genes that are operably linked to said promoter elements in the lactic acid bacteria from which said promoter elements are derived.

The latter feature distinguishes the claimed gene expression system from naturally occurring lactic acid bacteria which inherently comprise the promoter elements specified in the claim.

A gene expression system as defined in Claim 1 has neither been disclosed nor rendered obvious in any of the cited documents. The subject-matter of Claim 1 and of the claims which are directly or indirectly dependent thereon is therefore considered novel and also appears to involve the required inventive step (Article 33(2) and (3) EPC).

5. Claim 11 relates to a peptide which is defined by its amino acid sequence. The peptide is capable of activating the inducible promoter described in Claim 1. Said peptide has not been disclosed in the prior art and its sequence could not have been derived plainly and logically from any of the cited documents. Novelty and inventive step are therefore recognized for the subject-matter of Claim 11 (Article 33(2) and (3) PCT).

## CLAIMS

1. Gene expression system,  
**characterized in** that it comprises genes, promoter sequences and peptides involved in the production of bacteriocins except nisin in lactic acid bacteria.
- 5 2. Gene expression system,  
**characterized in** that it contains at least one specific regulated promoter, genes involved in transducing signals that induce gene expression, a peptide being that signal, and, possibly, genes involved in producing, processing, and secreting this inducing peptide.
- 10 3. The expression system of claims 1 and 2,  
**characterized in** that the said peptide is capable of inducing its own production and/or that of one or more bacteriocins by the lactic acid bacteria.
4. The expression system of claim 3,  
**characterized in** that said peptide is a functional analogue of the peptide of  
15 claim 3, functional analogues being defined as shortened, enlarged or mutated variants that retain the potential to induce gene expression.
5. The expression system of claim 4,  
wherein said peptide has the sequence Met-Ala-Gly-Asn-Ser-Ser-Asn-Phe-Ile-  
His-Lys-Ile-Lys-Gln-Ile-Phe-Thr-His-Arg, (seq. id. no. 1) or has the sequence  
20 Lys-Ser-Ser-Ala-Tyr-Ser-Leu-Gln-Met-Gly-Ala-Thr-Ala-Ile-Lys-Gln-Val-Lys-  
Lys-Leu-Phe-Lys-Lys-Trp-Gly-Trp (seq. id. no. 2).
6. The expression system of claim 1,  
**characterized in** that said promoter has a DNA sequence that is essentially  
similar to the promoter elements shown in Figure 4, essentially similar being  
25 defined by the presence of direct repeats, TATA boxes and a characteristic spacing between these elements and by the fact that expression initiated at this promoter can be induced by a mechanism similar to the mechanism for the induction of expression of the genes shown in Figure 1.
7. The expression system of claim 6, wherein said promoter has the DNA  
30 sequence of one of the promoter elements shown in Figure 4.

*Replaced by Article 3<sup>rd</sup>*

8. The expression system of claims 1-7,  
**characterized in** that one or more of the said genes are selected from the  
group of the genes denoted IF, K, R, P, I, T, A in Fig. 1, or the gene/genes  
are analogous of these genes, analogous genes being defined as genes that  
5 bear sequence homology with IF or K or R or P or I or T or A, are isolated  
from lactic acid bacteria, and are involved in bacteriocin production and/or  
the regulation thereof in those bacteria, and the said peptide is one of the  
peptides described in claims 3-5, and that the said promoter is one of the  
promoters described in claims 6 and 7.
- 10 9. The expression system of claim 1,  
**characterized in** that the said genes are at least one or more genes selected  
from the group the genes denoted IF, K, R, T, A, P, and I in Fig. 1, the said  
promoter has the DNA sequence of one of the promoters according to claim 6  
and 7 and the said peptide is Met-Ala-Gly-Asn-Ser-Ser-Asn-Phe-Ile-His-Lys-  
15 Ile-Lys-Gln-Ile-Phe-Thr-His-Arg (seq. id. no. 1), or any combination of two  
or more of the above genes.
10. A recombinant vector,  
**characterized in** comprising any possible combination of genes and promoter  
elements that are part of claim 1-9, preferably in that it contains a promoter  
20 element with the DNA sequence of one of the promoter elements described in  
claims 6 and 7 operably linked to a gene encoding a desired protein of  
interest.
11. A host cell,  
**characterized in** that it is transformed with the recombinant vector of claim  
25 10 and contains any possible combination of genes and promoter elements  
that are part of claims 1-9 integrated in the chromosome, and/or in that also  
integrated in its chromosome is a promoter element with the DNA sequence  
of one of the promoter elements described in claims 3 and 4 operably linked  
to an also integrated gene encoding a desired protein of interest.
- 30 12. The host cells of claim 11,  
**characterized in** that some of the said genes and promoter elements are  
present in plasmids and some are present in the chromosome.
13. The host cells of claims 11-12,

**characterized in** that the host is a Gram-positive bacterium, preferably a lactic acid bacterium.

14. The host cell of claims 11-13,  
**characterized in** that said host possesses the food consumption classification  
5 of GRAS (Generally Regarded As Safe).

15. The host cells of claims 11-14,  
**characterized in** that it is selected from the group consisting of members of  
the genera *Lactobacillus*, *Lactococcus*, *Pediococcus*, preferably members of  
the genus *Lactobacillus*, more preferably of *Lactobacillus sake* and  
10 *Lactobacillus plantarum*, most preferably of *Lactobacillus sake* LTH673 and  
*Lactobacillus plantarum* C11.

16. Purified peptide,  
**characterized in** that it has the amino acid sequence of Met-Ala-Gly-Asn-  
Ser-Ser-Asn-Phe-Ile-His-Lys-Ile-Lys-Gln-Ile-Phe-Thr-His-Arg (seq. id. no. 1).

15 17. Purified protein,  
**characterized in** that it is produced by any of the host cells of claims 11-15  
after or not addition of any of the peptides of claims 3-5.

18. Use the gene expression system according to claim 1-9, in any of the  
host cells described in claims 11-15 to induce gene expression by adding any  
20 of the peptides described in claims 3-5.

19. Use of any of the host cells of claims 11-15 in fermentations.

20. Use of any of the host cells of claims 11-15 to produce a desired protein  
of interest.

21. A kit for using the expression system according to claim 1, in lactic acid  
25 bacteria, **characterized in** consisting of:

1) One or more recombinant vectors each vector containing a promoter  
element identical or similar to one of the promoter elements depicted in Fig.  
4, directly followed by a multiple cloning site; these vectors may also contain  
one or more genes selected from the group K, R, IF, T, A (Fig. 1) or  
30 functional analogues of these genes,

2) Lactic acid bacteria that can function as host strain for these recombinant vectors, and that, depending on the recombinant vector used, may contain one or more genes selected from the group K, R, IF, T, A (Fig. 1) (or functional analogues of these genes) integrated in the chromosome,

- 5      3) A peptide that is capable of inducing the expression of genes under control of promoter elements similar or identical to the promoter elements depicted in Fig. 4 and that needs a two component system similar or identical to that encoded by genes K and R (Fig. 1) to exert its inducing action.

## CLAIMS

1. Gene expression system,  
characterized in that it comprises a gene/genes of interest that by genetic  
engineering have been operably linked to a strongly regulated promoter  
5 whose activity can be induced by an unmodified peptide, wherein said  
promoter and peptide are functional equivalent to promoters and peptides  
involved in the production of bacteriocins, except nisin, in lactic acid  
bacteria, and in that the products of two regulatory genes encoding a so  
called two-component regulatory system are essential for transducing the  
10 signal provided by said peptide into a change in activity of said strongly  
regulated promoter, and in that in naturally occurring lactic acid bacteria said  
regulatory genes are co-transcribed or closely associated with genes encoding  
said peptide, wherein the said peptide is a functional analogue of the peptide  
having the sequences shown in Seq. id. No. 1 and Seq. id. No. 2, and in that  
15 said gene/genes of interest are not identical to the genes that are operably  
linked to said promoter elements in the lactic acid bacterium from which said  
promoter elements are derived.
2. Gene expression system according to claim 1,  
characterized in that said peptide is capable of inducing its own production  
20 and/or the production of one or more bacteriocins in lactic acid bacteria.
3. Gene expression system according to claims 1-2,  
characterized in that said peptide is identical to the peptide having the  
sequences of Seq. id. No. 1 and Seq. id. No. 2.
4. Gene expression system according to claims 1-3,  
25 characterized in that said promoter is identical or functionally analogous to  
the promoter elements shown in Fig. 4.
5. A recombinant vector,  
characterized in that it comprises the gene/genes operably linked to the  
promoter elements according to claim 1-4, wherein this gene/these genes are  
30 not identical to the genes that are operably linked to said promoter elements  
in the lactic acid bacterium from which said promoter elements are derived.

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FORSKNINGSPARKEN A/S

6. A host cell,  
characterized in that it contains the gene/genes of claim 5 operably linked to the promoter, and in that the expression of the said gene/genes can be regulated by adding a peptide according to claims 1-4.
- 5 7. The host cells of claim 6,  
characterized in that some of the said genes and promoter elements are present in plasmids and some are present in the chromosome.
8. The host cells of claims 6-7,  
characterized in that the host is a Gram-positive bacterium, preferably a  
10 lactic acid bacterium.
9. The host cell of claims 6-8,  
characterized in that said host possesses the food consumption classification of GRAS (Generally Regarded As Safe).
10. The host cells of claims 6-9,  
15 characterized in that it is selected from the group consisting of members of the genera *Lactobacillus*, *Lactococcus*, *Pediococcus*, preferably members of the genus *Lactobacillus*, more preferably of *Lactobacillus sake* and *Lactobacillus plantarum*, most preferably of *Lactobacillus sake* LTH673 and *Lactobacillus plantarum* C11.
- 20 11. Peptide,  
characterized in that it has the amino acid sequence of Met-Ala-Gly-Asn-Ser-Ser-Asn-Phe-Ile-His-Lys-Ile-Lys-Gln-Ile-Phe-Thr-His-Arg (seq. id. no. 1).
12. Use the gene expression system according to claim 1-4, in any of the  
25 host cells described in claims 6-10 to induce gene expression by adding any of the peptides described in claim 3.
13. Use of any of the host cells of claims 6-10 in fermentations.
14. Use of any of the host cells of claims 6-10 to produce a desired protein of interest.

AMENDED SHEET

15. A kit for using the expression system according to claim 1, in lactic acid bacteria, characterized in consisting of:

5 1) One or more recombinant vectors each vector containing a promoter element identical or similar to one of the promoter elements depicted in Fig. 4, directly followed by a multiple cloning site; these vectors may also contain one or more genes selected from the group K, R, IF, T, A (Fig. 1) or functional analogues of these genes,

10 2) Lactic acid bacteria that can function as host strain for these recombinant vectors, and that, depending on the recombinant vector used, may contain one or more genes selected from the group K, R, IF, T, A (Fig. 1) (or functional analogues of these genes) integrated in the chromosome, such that at least the genes K and R or functional analogues thereof are present in said lactic acid bacteria containing said recombinant vector.

15 3) A peptide that is capable of inducing the expression of genes under control of promoter elements similar or identical to the promoter elements depicted in Fig. 4 and that needs a two component system similar or identical to that encoded by genes K and R (Fig. 1) to exert its inducing action.

AMENDED SHEET